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Primary active sodium transport, oxygen consumption, and ATP: Coupling and regulation

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The relationship between active transport at the plasma membrane and respiration has been studied extensively in the kidney, because this organ utilizes a large percentage of energy for this dissipative process. In the kidney, the rate of respiration follows linearly the rate of active transport. Various approaches have explored the coupling between these two variables [for review, 1, 2, 3]. To study this coupling, it has become necessary to investigate the properties of the mitochondria and the active transport systems within the intact cell. This has provided additional challenges not encountered when the mitochondria and the transport enzymes are studied in isolation. Ample evidence indicates that, in general, properties derived from isolated mitochondria and the isolated Na,K-ATPase are also displayed in the intact cell. However, some surprises have been forthcoming because of uncertainties concerning the cellular environment surrounding the mitochondria and transport enzymes.

This presentation is divided into the following four parts: cytosolic signal linking active transport with respiration, respiratory capacity of the mitochondria and active transport, differentiation between primary transport and primary metabolic events, and control of transport by metabolism.

Methods

Cytosolic signal linking active transport with respiration

The nature of the cytosolic signal linking changes in active transport to the accompanying alterations in respiration has been the subject of numerous investigations [1]. As shown in the schematic model for a proximal renal tubule (Fig. 1), ATP is hydrolyzed by the Na,K-ATPase into ADP and orthophosphate (P_i), which diffuse to the mitochondria for rephosphorylation into ATP. Alterations in the rate of Na,K-ATPase activity would be expected to result in concentration changes of ATP and/or its hydrolysis products [4, 5]. These products (ADP and P_i) have been shown to alter the respiratory rate in isolated mitochondria [6, 7]. Therefore, investigators have used experimental conditions which should be associated with changes in the cellular levels of ATP, ADP, and phosphate. As we shall see, the changes observed are qualitatively in the expected direction; however, quantitatively, these data are difficult to interpret unless there is compartmentation, particularly between the cytosol and the mitochondria.

About 6 years ago, Balaban et al [8], in my laboratory, measured the total ATP and ADP levels in a proximal tubule preparation after exposure to 25 μM ouabain in suspension under control conditions. While the total ATP levels changed little by ouabain addition, the ADP levels decreased by about 20%; the oxygen consumption was inhibited by about 50%. Qualitatively, this is the expected response because the lowered rate of ATP utilization elicited by ouabain would be expected to decrease the ADP levels, and thereby reduce ATP production and oxygen consumption. Whether ADP by itself constitutes the signal for respiratory control or whether it is the ATP/ADP ratio or the [ATP]/[ADP][P_i] ratio is a matter of intense controversy, which is beyond the scope of this review.

In isolated mitochondria, the dependence of respiration on the extramitochondrial concentration of ADP has been measured in the presence of excess P_i. A saturable function of ADP has been described with a K_m in the 15 to 50 μM range [9, 10]. If we take the ADP values of Balaban et al [8] and convert them into concentrations using a value of 2.4 μl/mg protein for cellular water [11], we obtain 520 μM for the control and 330 μM for ouabain. Both values would be clearly saturating for the mitochondrial ATP–ADP translocase [9] and thus the mitochondria respiration would be at their maximal respiratory rate (state 3) in both conditions. This quantitative discrepancy may be due to the difference between an average cellular concentration calculated from a total cellular extract and the *actual* free cytoplasmic concentration of ADP.

Various studies in other tissues suggest that most of the cellular ADP is compartmentalized within the mitochondria and, therefore, only a small percentage of the total ADP is free in the cytosol [10, 12, 13]. ADP has been reported to be concentrated by factors of 10 to 1,000 in the mitochondria [14]. Veech et al [10] found that only 5% of the total ADP in brain, liver, and muscle was metabolically active in the cytosol. If we assume the same proportion for kidney tubules, the control and ouabain-treated free ADP concentrations would be 32 and 21 μM, respectively, values close to the K_m for ADP in isolated mitochondria. This calculation agrees with ^{31}P NMR spectral analysis of rabbit and rat kidney tissue specimens [15, 16] as well as rabbit cortical tubules, showing that the free cytosolic ADP concentration is much lower than the ATP value. Although these calculations may not quantitatively represent the events occurring in the intact renal cell accurately, they do

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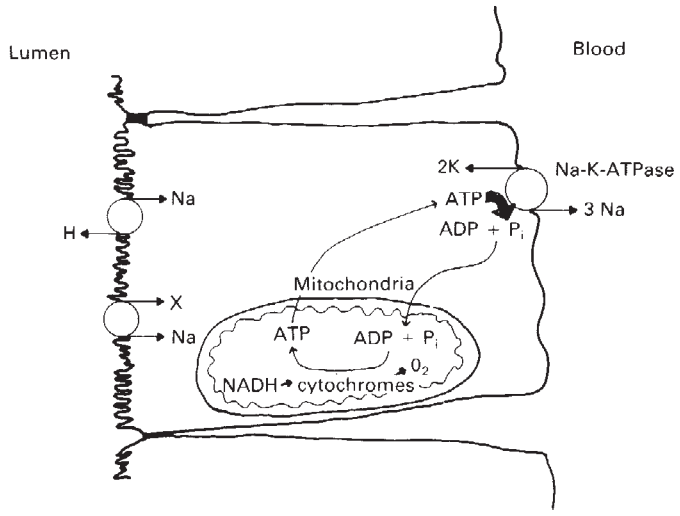

Fig. 1. *Schematic diagram of a renal proximal cell showing the relationship between mitochondrial ATP production and utilization of ATP by the Na,K-ATPase.*

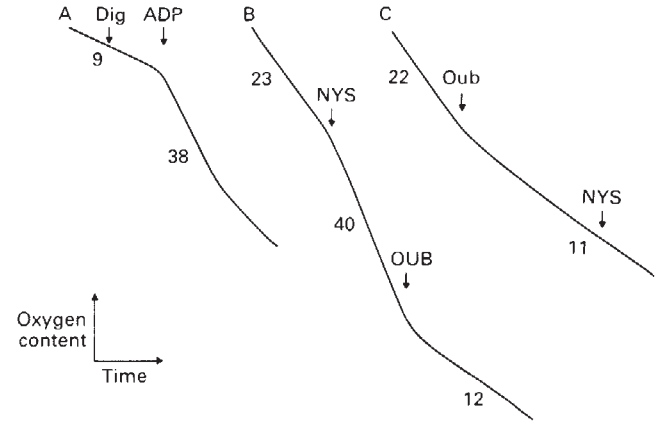

Fig. 2. *Oxygen consumption traces of renal proximal tubules.* The rate of consumption in nmoles O_2/min/mg protein is indicated by the numbers next to the traces. **A** Added digitonin (Dig), 0.1 mg/mg protein, ADP, 0.38 mM. **B** Added nystatin (Nys), 0.026 mg/mg protein; ouabain (Oub), 60 μM. **C** Added ouabain, 60 μM; nystatin 0.018 mg/mg protein. (*Adapted from* [21]).

indicate that the changes in ATP and ADP observed in this study during perturbations of active transport may explain the accompanying changes in oxygen consumption.

Respiratory capacity of the mitochondria and active transport

Measurement of the respiratory capacity (state 3 rate) of the in situ mitochondria provides important information on the maximal rate at which the mitochondria consume oxygen coupled to the production of ATP. In isolated mitochondria, this is obtained by the addition of a saturating concentration of ADP [17, 18]. Intact tubules are impermeant to ADP and, thus, to perform the experiment, the plasma membranes must first be permeabilized to ADP. In a proximal tubule suspension from the rabbit kidney, this was accomplished with the use of digitonin, which binds preferentially to cholesterol and, thus, at low concentrations seems to bind specifically to plasma membranes [19, 20]. Without digitonin, ADP has no effect on respiration. Figure 2 shows various traces of oxygen content versus time and the slopes, and measures the rates of oxygen consumption (qO_2), given by the numbers next to the traces. Trace A measures the qO_2 of tubules placed in KCl medium to approximate the intracellular ionic composition. In the absence of Na, the initial qO_2 is low and plasma membrane permeabilization with digitonin does not affect the qO_2 until ADP is added. ADP accelerates respiration by a factor of 4 or 5 to about 35 to 45 nmoles O_2/min · mg protein. This rate is termed the respiratory capacity of the mitochondria in the intact cell and is identical to the state 3 rate of mitochondria isolated from rabbit proximal tubules when both respiratory rates are normalized in terms of their cytochrome *a* content [21].

Table 1. Respiratory rate as compared to respiratory capacity in proximal tubules from the rabbit kidney

Experimental condition	qO_2 (*nmoles/min · mg protein*)	% Resp cap	ATP (*nmoles/mg protein*)
Respiratory capacity	44 ± 2	100	
Spontaneous rate	25 ± 2	56	9.8
Nystatin	44 ± 2	100	8.5
Ouabain	14	30	

This maximal rate was compared with oxygen consumption rates obtained in intact tubules bathed in a solution containing 150 mM Na, as well as other required salts and substrates, a situation close to that normally found in vivo. Under these conditions, normal respiration is about 20 to 25 nmoles O_2/min · mg protein (trace B), which is 50 to 60% of maximal. To determine how much respiration can be modulated by active Na transport, two extreme conditions were examined: maximal activation of Na,K-ATPase activity and total inhibition of the Na,K-ATPase. Maximal activation was obtained by the use of nystatin, a polyene antibiotic that makes the cells permeable to Na and K, equilibrating intra- and extracellular Na and K. This causes intracellular Na to increase from approximately 30 mM under normal conditions to 150 mM, which increases Na,K-ATPase activity, increasing the demand for ATP and causing a large increase in respiration, as shown in Figure 2. The effect of nystatin is only through the Na,K-ATPase, because it is fully inhibited by ouabain (trace C). If ouabain is added first, no effect of nystatin is observed.

Table 1 summarizes the results of a series of similar experiments. The respiratory capacity is 44 nmoles O_2/min · mg protein. If we define this as 100% of the ATP generating capacity of the tubular mitochondria, we see that the spontaneous rate of respiration is 56% of maximal. Stimulation of Na,K-ATPase activity with nystatin increases respiration to the maximum capacity and ouabain reduces respiration to 30% of capacity. Therefore, alterations in active transport could command up to 70% of the total respiratory capacity of the tubule [21]. These changes in respiration are accomplished with little, if any, change in ATP content in the presence of adequate substrates. With nystatin, ATP declines by only 15% and with ouabain, not shown here, ATP content increases by about 6% [8]. Therefore, it appears that the cell adjusts its respiration to the energetic demands and attempts to maintain a constant ATP level. Normally operating at 56% of capacity, the renal cell

would be expected to have much flexibility in responding to metabolic demands. This is indeed the case when the rate of active transport is altered. Another example of such an adjustment is obtained by stimulation of gluconeogenesis. The rate of glucose production can be increased two- to threefold by the addition of fatty acids butyrate and valerate, or by succinate. This is accomplished without affecting either the ATP content in the tubule suspension or fluid transport, measured in parallel experiments in isolated perfused proximal tubules from the rabbit [22].

These results demonstrate that the proximal renal cells have sufficient reserve capacity for energy production to maintain active transport and provide energy for other energy-requiring processes, such as gluconeogenesis. No evidence is found for energy limitations that would require sharing a limited energy supply. Quite the contrary, when sufficient metabolic substrate is supplied to the proximal tubules, there is enough energy for all dissipative processes. The renal cells adjust their rate of respiration to maintain the ATP levels approximately constant.

A similar situation seems to exist for the thick ascending limb (TAL). In a recently developed suspension of medullary TAL tubules from the rabbit, Chamberlin, LeFurgey, and Mandel [23] found that the addition of nystatin increased qO_2 by 108%. This result demonstrates the existence of ample respiratory capacity for this segment, too. The TALs show significant differences from proximal tubules in the metabolic support of active transport: First, the TALs use glucose as their main metabolic substrate [23], whereas proximal tubules function poorly when glucose is the only exogenous substrate [21]. Second, ouabain inhibits only 42% of the qO_2 in TALs [23], as compared to 50% for proximal tubules in the presence of glucose, lactate, and alanine. Third, the rate of respiration of TALs is much greater than that of proximal tubules. This translates into ouabain-sensitive qO_2s of 14 nmoles $O_2 \cdot$ mg protein$^{-1} \cdot$ min^{-1} for the TALs versus 9 nmoles $O_2 \cdot$ mg protein$^{-1} \cdot$ min^{-1} for the proximal tubules. Thus, the percentage of total oxygen consumption used for active transport is slightly lower in the thick ascending limb but the actual metabolic energy used per milligram of protein is 50% higher than in proximal tubules. This finding is not surprising because this tubule suspension has a greater activity of Na,K-ATPase than that reported for the suspension of proximal tubules [24].

The remaining metabolic energy (58% for TALs and 50% for proximal tubules) must be engaged in other energy-requiring activities besides active ion transport. The thick ascending limb has widespread endoplasmic reticula [25] suggesting that protein synthesis may be an important energy consuming process in the cell. Otherwise, to my knowledge, little information exists concerning the amount of energy that is required by the cells for non-transport events. This is clearly a potential area for fruitful future investigation.

Differentiation between primary transport and primary metabolic events

Combining qO_2 determinations with optical measurements of the redox level of the mitochondrial respiratory chain [26] allows for the differentiation of primary transport, primary metabolic events, or combinations of these two. In some cases, these methods can also be used to distinguish between effects on passive versus active transport. Numerous drugs, hormones, or other substances which affect transport in a tissue may have multiple sites of action and may, particularly, affect both metabolism and transport. For example, hormonal stimulation of energy-requiring processes often involves the simultaneous increase in substrate delivery rate and the rate of energy dissipation, as found in brown fat adipocytes [27]. Similarly, examples in transporting tissues are the initiation of acid secretion in the stomach, which is accompanied by substrate mobilization [28], and the stimulation of sodium transport by aldosterone on the toad bladder [29]. A few examples from work in my and associated laboratories will illustrate the use of these methods as applied to specific conditions in proximal kidney tubules:

(1) Amiloride is a known inhibitor of sodium entry in both tight and leaky epithelia [30]. In brushborder membranes from rabbit proximal tubules, it inhibits Na^+:H^+ exchange at concentrations of 10^{-4} to 10^{-3} M [31]. However, it is not clear whether at these concentrations the main inhibitory target of amiloride in the intact tubule is at the Na^+:H^+ exchanger. In a proximal tubule suspension, amiloride (10^{-3} M) inhibited the ouabain-sensitive oxygen consumption by up to 40% [32]. To identify the main inhibitory site, nystatin was added to bypass the entry step for sodium. Much to our surprise, amiloride [32] still inhibited qO_2 by up to 30%, suggesting a direct inhibitory effect on the Na,K-ATPase. Direct measurement of Na,K-ATPase enzyme activity in lysed proximal membranes confirmed this inhibition, indicating that the primary action of amiloride in the intact tubule was through inhibition of the Na,K-ATPase [32]. In more recent investigations, some amiloride analogs were found to also inhibit mitochondrial function (Soltoff, Mandel, and Cragoe, unpublished observations).

(2) Arsenate is a known inhibitor of phosphate reabsorption in proximal kidney tubules [33] and other tissues, but its mechanisms of inhibition are uncertain. We found that arsenate increased oxygen consumption and decreased NADH fluorescence, and these effects were magnified by the presence of ouabain [33]. These results indicated that the main effect of arsenate was to uncouple oxidative phosphorylation and that graded uncoupling of oxidative metabolism caused graded reductions in the net transport of both sodium and phosphate.

(3) Glucose promotes sodium transport by increasing luminal sodium entry via a sodium-solute cotransport mechanism [34, 35]. However, studies of in vivo glucose oxidation suggest that a selective linkage to sodium transport exists, involving glucose as a metabolic fuel for proximal fluid transport [2]. A recent study by Gullans, Harris, and Mandel [36] on a proximal tubule suspension clarifies this point. Glucose removal caused a decrease in qO_2 in the presence or absence of butyrate, a readily oxidizable fatty acid, suggesting that these qO_2 changes were related to the transport and not the metabolism of glucose. In addition, the nystatin-stimulated qO_2 was the same in the presence or absence of glucose. Finally, measurement of NADH fluorescence showed that the addition of glucose to a tubule suspension bathed in a glucose-free medium caused an oxidation of NAD. These data are all consistent with glucose acting to increase respiration by stimulating sodium entry at the luminal membrane (via glucose-sodium cotransport) followed

by increased sodium pump activity and its associated increase in mitochondrial respiration.

Control of transport by metabolism

For transepithelial transport, metabolic variables may influence the entry and/or exit rate of the transported substance through alterations in electrochemical gradient, membrane permeability, or pump activity. In a number of epithelial tissue specimens, investigators have attributed observed alterations in membrane permeability to factors as diverse as intracellular cyclic AMP [37], energy metabolism [38], calcium concentration [39], pH [40], and redox potential [41]. Furthermore, the effects of hormones, drugs, and pharmacological agents need to be superimposed on these variables. This array of potential effectors makes studying the control of transport by metabolism one of the most interesting but difficult areas of epithelial transport.

Cellular metabolism could also control the rate of active transport by alterations in the ATP levels or the phosphorylation potential. There is not much evidence that this occurs under normal conditions, because the cells tend to maintain a constant ATP level by adjusting respiration to the energetic demands of the cell, as was already seen for the kidney tubules. However, if alterations in cellular ATP level affected Na,K-ATPase activity, this could potentially be used by the cell as another control mechanism for active transport. Whether this does or does not occur in the healthy cell, decreased ATP levels have been observed clearly under pathological conditions.

The isolated Na,K-ATPase shows half-maximal stimulation as a function of ATP in the 0.3 to 0.5 mM range [42]. Total cellular ATP values calculated as concentrations normally are in the 3 to 8 mM range [8, 12, 13, 43] which would be expected to be saturating for the enzyme. Experimental conditions causing a 30 to 50% decline in ATP content would not be expected to affect Na,K-ATPase activity. However, much to our surprise, this expectation was not observed in intact tubules. A 30% inhibition of ATP content was obtained by using 10^{-7} M rotenone, which is a submaximal dose of this specific mitochondrial inhibitor [44]. The respiratory rate measured in the same tubule suspension was also inhibited by about 30%, resulting mainly from a decline in ouabain-sensitive respiration which is directly proportional to Na,K-ATPase activity. Parallel experiments performed in isolated perfused proximal convoluted tubules demonstrated that net sodium and fluid transport was also inhibited by about 30 to 40% in tubules exposed to 10^{-7} M rotenone, as compared with controls. Therefore, a condition that inhibited ATP production and caused a decline in ATP content of only 30% was associated with a proportional inhibition in Na,K-ATPase activity and transport [44]. Similarly, we observed [45] that phosphate depletion is associated with a 45% decrease in ATP levels as well as inhibited Na,K-ATPase activity and fluid transport. To investigate this surprising parallelism between ATP levels and active transport, we recently examined the dependence of Na,K-ATPase activity on total cellular ATP within the renal tubules [46].

A suspension of proximal tubules was exposed to graded amounts of rotenone, which inhibits the mitochondrial production of ATP by blocking the NADH dehydrogenase to lower the intracellular ATP content in a graded fashion. The tubules were initially suspended in a K^+-free medium to which rotenone was added, and the extracellular K concentration and the oxygen content were monitored on-line with electrodes. The extracellular potassium concentration measured at the beginning of the experiment was 0.2 to 0.3 mM, which is well below the $K_{1/2}$ of the Na,K-ATPase for potassium [47]. The Na,K-ATPase activity was stimulated 2 to 3 min after rotenone by the addition of a KCl bolus sufficient to raise the extracellular potassium concentration to about 5 mM. The KCl addition elicited a large increase in the respiratory rate, which was accompanied by the net uptake of potassium, measured by the disappearance of K from the medium. This K uptake represented active ion transport, because it was inhibited by ouabain and displayed kinetic properties that identified it as occurring through the Na,K-ATPase. Samples for ATP determinations were obtained during the period of maximal respiratory stimulation. These results enabled the rate of K uptake to be examined as a function of ATP in the intact tubules.

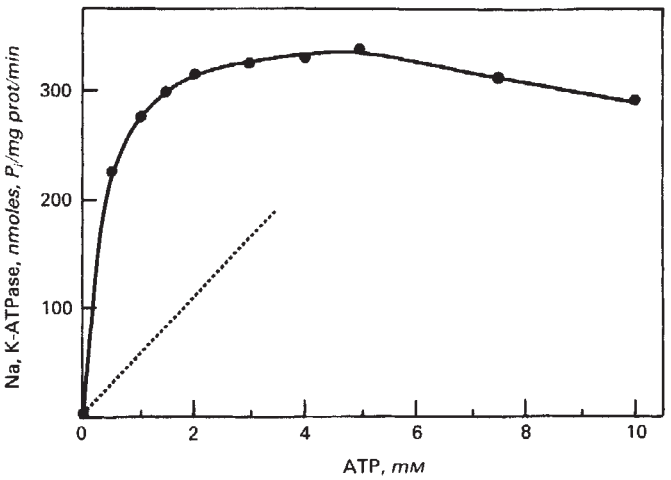

Fig. 3. *The dependence of the Na,K-ATPase activity of proximal tubule membranes* (solid line) *and the sodium pump activity of intact proximal tubules* (broken line) *on the ATP concentration. (Reproduced with permission from* [49]).

Interestingly, the potassium uptake rate was a linear function of the ATP content. The intracellular Na^+ concentration was about 150 mM during the K^+-free incubation and presumably saturated the intracellular Na^+ site of the ATPase, so these results singularly reflected the dependence of the Na,K-ATPase on ATP during conditions of maximal stimulation.

The ATP dependence of the Na,K-ATPase (sodium pump) activity of the intact proximal tubule could be compared quantitatively to that of the Na,K-ATPase of the membrane preparation by assuming a K/ATP stoichiometry of 2 [47].

The Na,K-ATPase activities of both preparations as a function of ATP concentration are shown in Figure 3. In these experiments, the maximal Na,K-ATPase activity in the intact tubules was obtained without rotenone thus with ATP concentrations in the 3 to 4 mM range (Fig. 3, highest point on dotted line). Lower ATP values were obtained with the graded rotenone additions. Qualitatively, these results provide important information regarding a novel concept, namely, that the cells normally do not possess a saturating concentration of ATP, suggesting that cellular metabolism may play a controlling role in the regulation of sodium transport.

In these studies, it is quantitatively not possible to evaluate the ATP dependence in terms of alterations in a $K_{1/2}$ value, since the sodium pump is not saturated with ATP in the intact tubule. Nevertheless, the affinity of the sodium pump for ATP appears to be much lower than that of the Na,K-ATPase measured in the lysed membrane preparation. Moreover, the maximum Na,K-ATPase activity (~340 nmoles P_i/mg protein · min) for the proximal tubule membranes was approximately twice the calculated maximum observed for the Na,K-ATPase activity (~185 nmoles P_i/mg protein · min) of the tubules in suspension (Fig. 3). Thus, there are real quantitative differences between the results obtained in this study using the lysed membranes under specific well-defined assay conditions, and those obtained using a preparation that may be regulated in a more physiological manner. In the intact tubule, it is possible that the microenvironment in the vicinity of the Na,K-ATPase may be different from the one surrounding the lysed membrane fragments. The local concentrations of ATP, ADP, phosphate, magnesium, or vanadate in the vicinity of the pump are unknown. These compounds, singularly or in combination, could affect the kinetic properties of the Na pump [46].

Another important metabolic variable that has been shown to affect transport is phosphate, which became particularly apparent in experiments with phosphate-deprived tubules. In isolated perfused proximal convoluted tubules, perfusion of the tubular lumen with a fluid containing no phosphate completely inhibited fluid transport [45]. In parallel experiments performed in a proximal tubule suspension, the removal of extracellular phosphate produced appreciable inhibitions in both the control oxygen consumption as well as the qO_2 stimulated by respiratory uncouplers. In the perfused tubule experiments, the effect of phosphate depletion could only be elicited from the lumen, because removal of phosphate from the bath did not inhibit fluid transport (J_v). Thus, phosphate entry across the luminal membrane may be an important source of inorganic phosphate for cellular metabolism [45].

In this regard, the effect of sugars on the inhibitory actions of the P_i-free medium was very interesting, particularly the observation that the inhibition of J_v required the presence of glucose in the perfusate [45]. A series of experiments examined whether it was the entry of glucose into the cells or the metabolism of glucose that was responsible for this inhibition of fluid transport. Phlorizin, which inhibits the entry of glucose through the luminal membrane, maintained J_v in a phosphate-free medium. Thus, the prevention of glucose entry protected the cells from the inhibiting action of glucose in phosphate-free medium. Similarly, substitution of α-methylglucoside (a sugar that is transported but not metabolized) for glucose also protected the tubule and maintained a normal J_v. Finally, the addition to the bath of 2-deoxyglucose, which blocks glucose metabolism, had a similar protective action.

These results suggest that the entry of glucose through the apical side followed by its metabolism interacts with intracellular phosphate to inhibit oxidative metabolism and transport. The pattern is similar to the Crabtree effect in which phosphate is sequestered by metabolic intermediates of glycolysis in the cytosol and therefore becomes limiting for oxidative metabolism [45].

Under these phosphate-deprived conditions, phosphate could become limiting to oxidative metabolism in at least two ways:

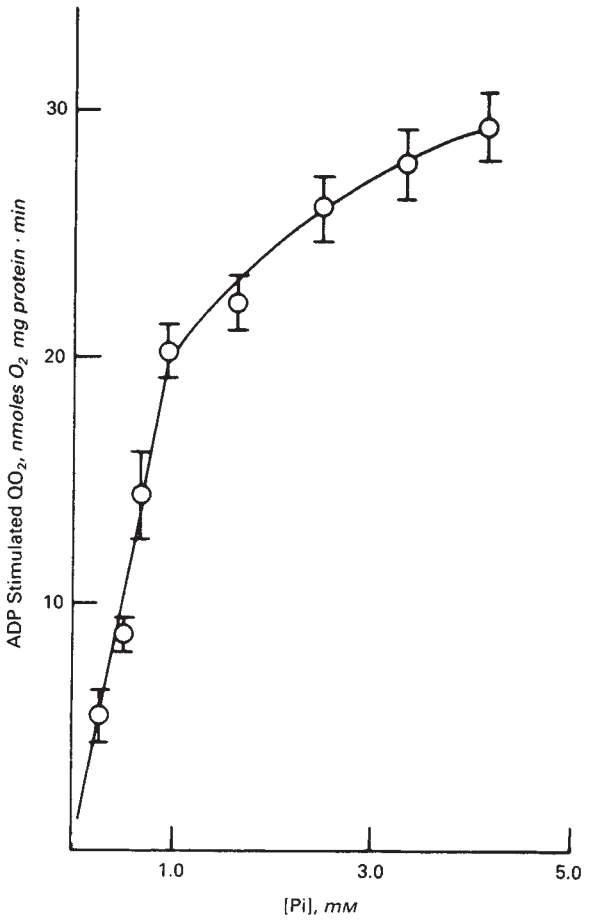

Fig. 4. *Dependence of ATP-stimulated oxygen consumption (State 3 respiration) on cytosolic phosphate of proximal rabbit tubules.* The experiments were performed in four to seven suspensions of digitonin-treated tubules. (*Reproduced with permission from* [52]).

(1) directly, since P_i is a substrate of oxidative phosphorylation, reacting with ADP to form ATP; or *(2)* indirectly, since intramitochondrial P_i exchanges for dicarboxylic acids that are Krebs cycle intermediates [14]. Therefore, the phosphate concentration in the mitochondrial matrix could be expected to influence the mitochondrial level of these intermediates. We examined the direct dependence of oxidative phosphorylation on cytoplasmic P_i by measuring the state 3 response of the tubule mitochondria as a function of P_i. This experiment was performed with tubules in suspension bathed in a KCl medium. Digitonin was added to permeabilize the plasma membranes to ADP and P_i, and a maximal ADP concentration was added in the presence of varying concentrations of P_i. The results (Fig. 4) showed a linear dependence on P_i below 1 mM, and a tendency toward saturation at higher P_i values [48]. How do these values compare with the normal free cytosolic concentration of phosphate? In the presence of normal extracellular phosphate, the total phosphate content approximates 5 mM, but most of this is bound [12]. Using NMR measurements, Freeman et al [49] estimated the free P_i to be about 0.6 mM. Such a low value would suggest that P_i is normally limiting for oxidative phosphorylation and that this effect would definitely be magnified further by P_i depletion.

Summary

Several metabolic aspects of primary active transport have been explored in this communication. One emphasized theme entailed the need to investigate the properties of the mitochondria and the active transport systems within the intact cell. Several methodological and conceptual approaches were described that permitted such an analysis. The answers provided were sometimes qualitative or quantitative. Qualitative information was provided regarding the cytosolic signal linking active transport with respiration, suggesting that the cytosolic ADP concentration was an important element in that link. The intact renal cell was found to work normally at 50 to 60% of its maximal respiratory capacity, indicating that sufficient reserve capacity was present for increased metabolic demands.

Several examples were described in which a combination of qO_2 measurements and/or optical techniques were used to differentiate between effects of agents which act primarily on transport or metabolic events. Finally, the control of transport by metabolism was discussed, primarily emphasizing the role of ATP and P_i. One of the overall conclusions from these studies is that, in general, the mitochondria and the transport systems seem to display similar properties in the intact cell as they do in isolated form. However, uncertainties concerning the cellular microenvironment surrounding the mitochondria and the plasma membrane transporters have produced some interesting surprises concerning their function in the intact cell. More quantitative information on the energy compartmentation of the renal cell would be helpful to clarify numerous aspects of metabolic function.

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